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TITLE: Breast Cancer Prevention by Hormonally Induced Mammary  
Gland Differentiation: The Role of a Novel Mammary  
Growth Inhibitor and Differentiation Factor MRG

PRINCIPAL INVESTIGATOR: Yuenian Eric Shi, M.D., Ph.D.

CONTRACTING ORGANIZATION: Long Island Jewish Medical Center  
New Hyde Park, New York 11040

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<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> Long Island Jewish Medical Center New Hyde Park, New York 11040  <i>E-Mail:</i> shi@lij.edu			<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
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## I. INTRODUCTION

**I-1. Mammary derived growth inhibitor (MDGI) Related Gene MRG.** Mammary gland development is controlled by systemic hormones and by local growth factors that might complement or mediate hormonal actions. In an effort to search growth regulators in the human mammary gland, we generated cDNA libraries from a breast cancer biopsy specimen and a normal breast and analyzed these libraries by differential cDNA sequencing (1). We identified, cloned, and characterized a novel tumor growth inhibitor and named it the Mammary derived growth inhibitor-Related Gene MRG (2). The predicted amino acid sequence of MRG has a significant sequence homology to previously identified mouse mammary derived growth inhibitor **MDGI** (3). MDGI is a mammary epithelial cell growth inhibitor and differentiation factor initially identified and purified from Ehrlich ascites mammary carcinoma cells (3), and then from the lactating bovine mammary gland (4-5) and from cows milk (6). Studies of **mouse and bovine** MDGI suggest several functions of MDGI on growth and differentiation of mammary gland. MDGI specifically inhibit the growth of normal mouse mammary epithelial cells (MEC), and promote morphological differentiation: the appearance of bulbous alveolar end buds and formation of fully developed lobuloalveolar structures (7). Selective inhibition of endogenous MDGI expression in mouse MEC by use of antisense oligonucleotides suppresses alveolar budding and impairs  $\beta$ -casein synthesis in organ cultures (7). Increasing amounts of MDGI mRNA were detected in terminal parts of ducts and lobuloalveolar epithelial cells of differentiated glands and maximally expressed in the terminally differentiated state found just prior to lactation (8). MDGI expression in mouse mammary epithelium cells is hormonally regulated (9-10). Many of these growth inhibition and differentiating effects of MDGI are **conserved** in MRG.

**II-2. Fatty acid binding protein (FABP).** Interestingly, MRG and MDGI revealed no homology to any other known growth inhibitors; rather, they revealed extensive sequence homology to FABP (11-12). A striking homology was evident between bovine MDGI and Heart type (H-) FABP, which differ only in seven positions of the amino acid sequence (13). In fact, it turned out that the originally described MDGI is a mix of H-FABP and adipocyte type (A-) FABP both expressed in mammary gland (14-15). H-FABP fully replaced the MDGI effect and inhibited the growth of mammary epithelial cells (14). Like MDGI and H-FABP, the sequence of MRG was found to be identical to the recently deposited sequences of human brain type (**B-**) FABP in GenBank (accession #AJ002962) (12). Cellular FABPs are a highly conserved family of proteins consisting of several subtypes and have been suggested to be involved in intracellular fatty acid metabolism and trafficking. Among them, only H-FABP/MDGI and the recently identified B-FABP/MRG have a differentiating effect on mammary epithelial cells and tumor suppressing activity against breast cancer. In this regard, we suggest to keep the names of MDGI and MRG when referring their functions on mammary gland and use H-FABP and B-FABP when referring their well-accepted FABP family phylogenetic tree (12).

**I-3. The roles of MRG/B-FABP on mammary gland differentiation and suppression of breast cancer growth.** FABPs comprise a well-established family of **cytoplasmic** hydrophobic ligand binding proteins and are thought to be involved in lipid metabolism by binding and intracellular transport of long-chain fatty acids. However, from other studies on role for FABPs in cell signaling, growth inhibition and differentiation has also been implied (12,16-17). In particular, H-FABP and B-FABP are abundantly expressed in differentiated mammary gland. It has been suggested that in heart and brain, FABPs regulate the supply of fatty acids to the mitochondria for beta-oxidation (18-19). The mammary gland, however, is a highly **lipogenic** tissue and fatty acids are not likely to be a major fuel for its metabolism. Within the phylogenetic tree of FABPs, B-FABP and H-FABP belong to a closely related subfamily of proteins that act as tumor suppressors for breast cancer (12). Therefore, MRG and MDGI could fulfill different functions in brain and heart compared with mammary gland.

MDGI/H-FABP protein was mainly detected in myocardium, skeletal and smooth muscle fibres, lipid, and steroid synthesizing cells adrenals, lactating mammary gland, and terminally differentiated epithelia of the respiratory, intestinal and urogenital tracts (20). Within the similar content, the expression of MRG was mainly detected in **brain, heart, and skeletal muscle**, which are in the postmitotic status (2). Abundant MRG protein expression was also detected in human lactating mammary epithelial cells by immunohistochemical staining (21). These results provide evidence that expression of MRG and MDGI are associated with an irreversibly **postmitotic and terminally differentiated** status of cells. It has been previously demonstrated that the expression of B-FABP (mouse MRG) is correlated with neuronal differentiation in many parts of the mouse central nervous system (22-23) and blocking antibody to B-FABP can block glial cell differentiation in mixed primary cell cultures prepared during the first postnatal week (22). In mammary epithelium, MRG also induces mammary differentiation (21). These include that (a) overexpression of MRG in human breast cancer cells induced differentiated cellular morphology and a significant increase in the production of lipid droplets and (b) treatment of mouse mammary gland in organ culture with MRGp resulted in a differentiated morphology and production of  $\beta$ -casein (Appendix 1). Therefore, it seems clear that a differentiation-associated function is a common property of these structurally related subfamily of FABPs. Being the members of FABP family, the most characterized biological functions for MRG/B-FABP are tumor suppressing activities against breast cancer and differentiating effect on mammary cells. These include:

- 1). The loss of B-FABP/MRG expression (2) and H-FABP/MDGI (24) is associated with breast cancer progression.
- 2). Both MRG (21) and MDGI (11,25) are highly expressed in the fully differentiated lactating mammary gland and induce mammary differentiation.
- 3). MRG and MDGI have been mapped at the chromosome 6q22-23 (12) and 1p35 (26) that harbor the putative tumor suppressor genes for breast cancer (27-28).
- 4). Both MRG and MDGI strongly suppress the growth of breast tumors (2,26).

#### **I-4. Hypotheses**

1. MRG is a candidate mediator of the differentiating effect of pregnancy and lactation on breast epithelial cells; its expression is maximal in the differentiated state and hormonally regulated.
2. Up-regulation of MRG expression in the young nulliparous MRG transgenic female mice can mimic pregnancy-and lactation-induced mammary gland differentiation and prevent breast cancer incidence.

#### **I-5. Specific aims**

**SA1.** MRG expression and hormonal regulation during mammary gland differentiation and lactation. We will first confirm and extend preliminary studies, which suggest that MRG is a differentiation factor and its expression is associated with mammary gland differentiation and lactation. We will then determine if differentiation-related regulation of MRG expression is specific to hormonal regulation during pregnancy and lactation or MRG in general is also regulated by non-hormone associated differentiating agents.

**SA2.** Prevention of breast cancer in MRG transgenic mice. We will determine if overexpression of MRG in the transgenic mice will prevent breast cancer induced by carcinogen 7,12-Dimethylbenz-[a]-anthracene (DMBA). We also compare the pregnancy- and lactation-induced prevention vs. the MRG-induced prevention.

## **II. WORK ACCOMPLISHED**

**II-1. Specific Aim 1:** MRG expression and hormonal regulation. FINISHED (Cancer Res, Appendix 1)

A. Screening of MRG expression in clinical breast specimens. In an attempt to evaluate the potential biological significance of MRG on differentiation and lactation of human mammary gland, we studied MRG protein expression in the formalin-fixed and paraffin-embedded clinical human biopsy specimens from normal breast reduction mammoplasty specimens, lactating mammary glands, and malignant breast carcinomas. As shown in the Fig.1 of the Cancer Res paper, we found a strongly positive MRG protein staining in the alveolar mammary epithelial cells from the lactating mammary gland. The expression of MRG protein was clearly detectable in the alveolar epithelial cells in all 5 lactating mammary glands. In contrast, either no detectable MRG protein staining or very weak MRG protein expression was visualized in 8 cases of the non-pregnant normal breast reduction mammoplasty specimens from nulliparous women. Expression of MRG protein was absent in all 10 cases of malignant breast carcinomas.

B. Effects of MRG overexpression on the expression of differentiation-related milk protein genes. To investigate if the high level of MRG expression in the lactating alveolar mammary epithelial is an instigator or merely a by-product during mammary gland differentiation leading to the milk production, we investigated whether overexpression of MRG gene could induce differentiation. We transfected MDA-MB-231 human breast cancer cells with full-length MRG cDNA and established several MRG expressing clones (MRG-231 clones) (1). Fig. 3A shows the MRG protein expression in MRG-231-10 and MRG-231-6 cells, two MRG positive clones, but not in parental MDA-MB-231 and neo-231-1 MRG negative cells.

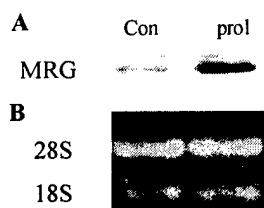
It is well established that the extracellular matrix is required for normal functional differentiation of mammary epithelia. Striking changes in cell morphology were observed when MRG-231 cells were cultured in the Matrigel coated dish. MRG-231-10 cells were aggregated to form spheroids on a reconstituted basement membrane gel (Fig. 3B), a typical differentiated phenotype for mammary epithelial cells (28). In contrast, neo-231-1 cells showed considerable heterogeneity in cell size, and many cells had "fibroblast-like" spreading morphology (Fig.3C).

We examined whether MRG-induced morphological changes are consistent with differentiation. Because the maturation of breast cells is characterized by the presence of lipid droplets that are milk components, we examined the lipid accumulation on MRG-231 cells compared with the control cells. Droplets containing neutral lipid were readily detectable in MRG-231-6 clones cultured in the non-coated culture plates; in contrast, no obvious lipid droplet could be observed in the neo-231-1 cells. When the lipid-producing cells were counted, 2 % and 5 % of MRG-231-6 and MRG-231-10 cells produced lipid droplets, respectively, but virtually no lipid producing cells were observed in MDA-MB-231 and neo-231-1 cells. When the cells cultured in the Matrigel-coated plates, a significant increase in lipid accumulation was observed in both MRG-231 cells and MRG negative control cells. Representatives of lipid staining in MRG-231-6 and neo-231-1 cells were shown in Fig. 4. Fifteen % of MRG-231-6 and 21% of MRG-231-10 cells produced lipid droplets, but only 4 % of MDA-MB-231 cells and 3 % of neo-231-1 contained lipid droplets, which were much smaller size than that of MRG positive cells (Table 1).

Induction of differentiation of mouse mammary gland by MRG recombinant protein (MRGp). Tissue-specific expression of milk protein in mammary epithelial cells depends on contact with stromal cells and matrix proteins. To further confirm the differentiating effect of MRG on mammary gland, we used the mouse whole-organ culture of mammary gland to study whether MRGp can regulate milk protein  $\beta$ -casein. The glands from virgin mice were cultured for 6 days with or without 50 nM MRGp. In mammary gland development, the alveolar buds represent a developmental pathway that eventually leads to secretory alveoli during functional differentiation. Histological examination of MRGp-treated glands revealed the appearance of secretory active alveoli with enlarged luminal spaces and the induction of lipid accumulation (Fig. 5, A & B). In consistent with these changes, which are

characteristic for the differentiated phenotype, functional differentiation with stimulation of  $\beta$ -casein was also observed. While no detectable  $\beta$ -casein mRNA was observed in control mammary glands, expression of  $\beta$ -casein mRNA was significantly increased in MRGp treated glands (Fig. 5, C & D). Therefore, treatment of mouse mammary gland in organ culture with MRGp resulted in a histologically differentiated phenotype as well as functional differentiation.

**C. Regulation of MRG expression by hormones. Stimulation of MRG expression by prolactin.** Since mammary differentiation is controlled by systemic hormones, we were interested to see whether MRG expression is regulated by the hormones such as prolactin. In this regard, we tested effects of prolactin on MRG expression in T47D cells. Treatment of the cells with prolactin resulted in a 5.8-fold increase in the MRG expression (Fig. 1).

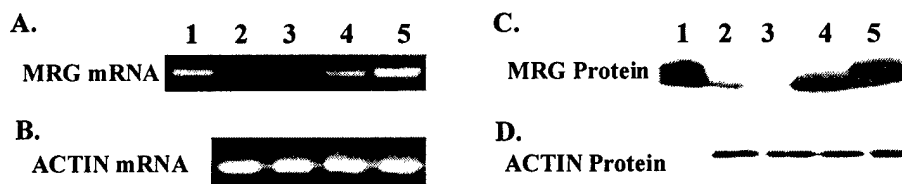


**Fig. 1.** Stimulation of MRG expression by prolactin. T47D cells were culture collagen coated dishes in DMEM containing 5% FCS and 5  $\mu$ g/ml of insulin. Cells were treated with or without 80 IU of prolactin for 12 hr. Total RNA was isolated and analyzed (20 mg/lane) by Northern blot. The integrity and the loading control of the RNAs were ascertained by direct visualization of the 28 S and 18 S rRNA in stained gel.

## II-2. Specific Aim 2: Prevention of breast cancer in MRG transgenic mice.

**A. To generate transgenic mice overexpressing human MRG under the control of MMTV promoter (FINISHED)** (J. Biol. Chem. 278: 47319-47325, 2003)

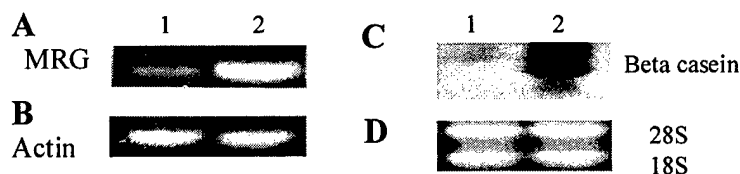
**A1. Screening, identification, and maintenance of mice heterozygous and homozygous for the transgene.** Mating founder animals to wild-type (FVB/n background) males and females generated four 1<sup>st</sup>-generation transgenic lines. Transgenic males and females from the same family were mated to generate homozygous mice. If a mouse produced two or more litters of offspring that were transgenic, the mouse was considered to carry the transgene. Homozygous male and female mice from the same family were mated to each other to maintain the homozygous lines. Among the four lines, MRG mRNA expressions in mammary gland was highest in family of MM16, and progressively lower levels of MRG expression were observed in families of MM4 and MM3. Two homozygous MMTV/MRG lines from MM16 and MM4 families were generated and named as MM-H1 and MM-H2. Fig. 2 shows the transgene mRNA and protein expression in these two homozygous lines as well as two control littermates. As expected, the transgene and its protein were highly expressed in MM-H1 and MM-H2 lines. Since the transgene is driven by the MMTV promoter, which is maximally active during the pregnancy, the transgene expression could be further increased. However, as we demonstrated in Fig. 3, the expression of endogenous mouse MRG/B-FABP in pregnant mammary gland also increased considerably.



**Fig. 2.** MRG transgene expression in control and homozygous transgenic lines. Eight-week old virgin MM-H1 and MM-H2 mice, and age matched control virgin mice were scarified and the third pare

thoracic mammary glands were removed. The left gland was subjected to RNA isolation and RT-PCR analysis and the right gland was subjected protein isolation and Western analysis. (A). RT-PCR analysis of MRG using primers within MRG coding sequence (5'-GTGGAGGCTTTCTGTGCTACCTGG-3' and 5'-TGCCTTCTCATAGTGGCGAACAG-3'). The 393-bp PCR product is a specific indication of the presence of **human** MRG transgene. The integrity and the loading control of the RNA samples were ascertained by actin expression with a set of primers (5'-GCTGTGCTATCCCTGTACGC-3' and 5'-TGCCTCAGGGCAGCGGAACC-3') for 314-bp  $\beta$ -actin (B). Lane 1, T47D cells as a positive control; lane 2-3, control mice; lane 4, MM-H2; lane 5, MM-H1. Each reaction consisted of 25 cycles in the GeneAmp PCR System 2400 (Perkin Elmer). The parameters for PCR were: denaturation at 94°C for 30 s; annealing and elongation at 55°C for 30 s. and at 72°C for 30 s. One third of the PCR products were electrophoresed through 1% agarose-TAE-gel. (C-D). Western analysis of MRG protein and actin expression. Western blot using the specific anti-MRG antibody was carried out as we previously described. Lane 1, 10 ng of purified recombinant MRG protein; lane 2-3, control mice; lane 4, MM-H2; lane 5, MM-H2. Lanes 2-5 contained 50  $\mu$ g of cellular protein. Please note that our antibody did cross-react with mouse MRG.

**A2. Expression of endogenous mouse MRG/B-FABP in mammary gland of control mice.** To address the role of endogenous versus the transgenic MRG in breast epithelial differentiation, we analyzed the endogenous MRG expression in control virgin mouse vs. control pregnant mouse by RT-PCR (Fig. 3). As expected, the 550-bp endogenous **mouse** MRG was clearly present in the mammary gland during pregnancy. However, there was a **very weak** endogenous mouse MRG expression in the gland from non-pregnant virgin mouse. In a similar pattern, while expression of  $\beta$ -casein was abundant in the gland from pregnant mouse, it was barely detectable in the gland from control virgin mouse.



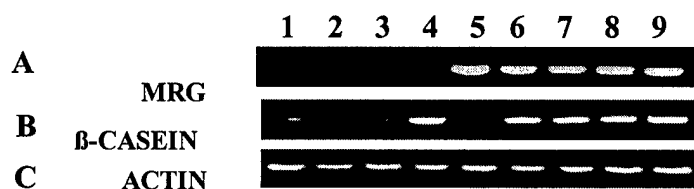
**Fig. 3.** Expression of mouse MRG/B-FABP and  $\beta$ -casein in control non-transgenic mice. Third thoracic mammary glands were isolated from 9-week old non-transgenic virgin and pregnant mice. Expression of mouse MRG mRNA (A) was analyzed by RT-PCR and normalized for  $\beta$ -actin expression (B). Four  $\mu$ g total RNA were used for RT reaction using a First-Strand cDNA synthesis kit (Boehringer Mannheim), and one tenth of this reaction was used for the PCR. The 550-bp of the mouse MRG gene was amplified by PCR with a set of primers (5'TGG TAG ATG CTT TCT GCG CA-3' and 5'TCA AAA GCA AGT TCC CAT TCA A-3'). To control for cDNA quality and quantity, a 314-bp  $\beta$ -actin fragment was analyzed. Densitometric scan indicates that MRG expression is increased 10-fold during pregnancy. Expression of  $\beta$ -casein was analyzed by Northern blot (C) and normalized by direct visualization of the ribosomal RNAs in stained gel (D).

#### **B. To determine if the effects of MRG overexpression on mammary gland development.**

**B1. Stimulation of  $\beta$ -casein expression.** To determine if the expressed transgene stimulates the functional differentiation, we developed a quick screening assay for analysis of MRG and  $\beta$ -casein expression by RT-PCR. Fig. 4 shows a representative MRG transgene and  $\beta$ -casein expression in four virgin control mice and four randomly picked fourth generation virgin transgenic mice from MM-H1 and MM-H2 lines. While control mice did not have the transgene, all picked four transgenic pups had



transgene expression. Most importantly, all four transgenic mice also have  $\beta$ -casein expression, which was not detectable in control virgin mice. These results indicate that the mammary glands of the established MMTV/MRG transgenic lines MM-H1 and MM-H2 have functional expression of the transgene, which stimulates mammary gland differentiation.

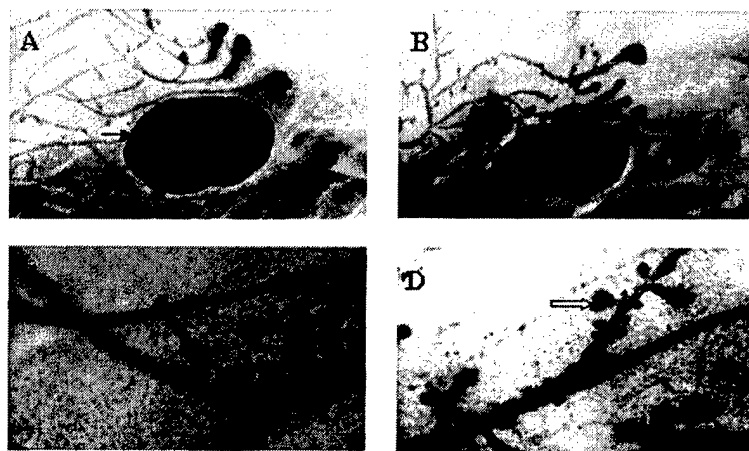


**Fig. 4.** RT-PCR analysis of MRG transgene and  $\beta$ -casein expression. Eight-week old fourth generation virgin MM-H1 and MM-H2 mice, and age matched control virgin mice and control pregnant mouse were scarified and the third pair thoracic mammary glands were removed.

Expression of MRG transgene (A) and  $\beta$ -casein mRNA (B) was analyzed by RT-PCR and normalized for  $\beta$ -actin expression (C). RNA from T47D cells was used as a positive control for MRG expression (lane 5). RT-PCR was conducted as described in Fig. 3. The 393-bp of the human MRG was amplified by PCR with a set of primer as described in Fig. 2. The 480-bp of the mouse  $\beta$ -casein gene was amplified by PCR with a set of primers (5'-GTC TCT TCC TCA GTC CAA AGT-3' and 5'-TTG AAA TGA CTG GAA AGG AAA TAG-3'). **Lanes 1-4**, control mice; **lane 4**, control pregnant mouse; **lane 5**, T47D breast cancer cell; **lane 6**, MM-H1 #2; **lane 7**, MM-H1 #4; **lane 8**, MM-H2 #1, **lane 9**, MM-H2 #2.

## B2. Induction of differentiated gland morphology with increased lobulo-alveoli in the gland from the transgenic line.

Because MRG protein expression was associated with human mammary gland differentiation with the highest expression observed in the differentiated alveolar mammary epithelial cells from the lactating gland, we were interested in studying whether MRG is an instigator of mammary gland differentiation or merely a correlative product during mammary gland development. The effect of transgene expression on mammary gland development and functional differentiation was assayed by morphological analyses of ductal elongation and appearance of a differentiated alveolar branching morphogenesis. While the mammary gland development starts at about 3-week old in wild-type mice with ductal elongation and development of the initial branching structure, the functional differentiation starts at the onset of pregnancy with the expansion of secretory lobulo-alveolar architect. Whole mount preparations of the mammary glands from virgin wild-type and virgin transgenic mice were examined to determine the effect of MRG on mammary gland development. Fig. 5 shows a representative mammary gland analysis of 40-day old transgenic mouse vs. wild-type control littermate. Mammary ducts in the transgenic virgin as well as in the control virgin littermate filled the typical  $\frac{1}{2}$  length of the

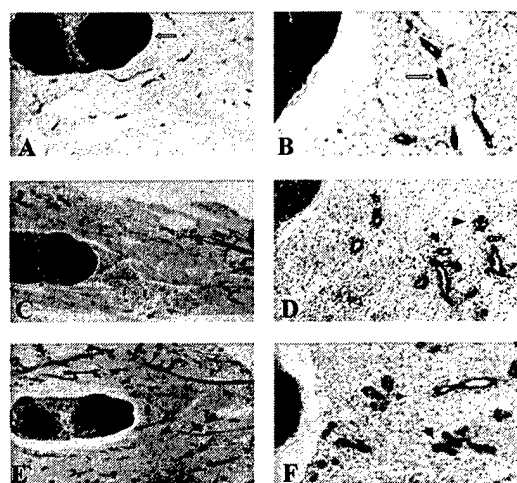


inguinal gland and appeared normal (Fig. 5, compare A and B), indicating that expression of the transgene did not alter the ductal outgrowth during the early mammary gland development. However, an alternation in the developmental pattern of the distal cells of ducts in transgenic virgin mice (Fig. 5D) was observed compared with the control littermate (Fig. 5C). While the limited budding was developed in the wild-type gland (Fig. 5C), transgenic gland exhibited multiplicity

of budding (Fig. 5D).

**Fig. 5.** Whole mount histological analysis of mammary gland from female MM-H2 transgenic mouse and wild-type littermate. A 40-day old virgin MM-H2 mouse and a age-matched virgin wild-type littermate mouse were sacrificed, the right inguinal gland were removed and subjected to whole mount gland fix, defat, and staining. **A & C**, wild-type control mouse. **B & D**, MM-H2 transgenic mouse. **A & B**, lower magnification images from (Nikon, 2X10). Arrows indicate the inguinal lymph node and the direction for duct extension (from left to right). **C & D**, higher magnification pictures from (10X10). An open arrow indicates budding.

Using whole mount histological analysis, we performed a histological analysis of formation of lobulo-alveoli. As shown in **Fig. 6**, while there is limited lobulo-alveolar structure in the 7-week old control virgin mice (**A & B**), a significant increase in the formation of lobulo-alveolar structure was observed in the gland from MMTV/MRG mice (**C & D**). Giving the fact that mammary gland development and differentiation is controlled by **systemic hormones** and by a variety of different local growth factors that might complement or mediate hormonal actions, we are interested in comparison of the magnitude of this MRG-induced formation of alveoli to that of hormone stimulated alveoli formation. As we mentioned in the grant (p27), Russo has demonstrated that treatment of rat with human placental hormone chorionic gonadotropin (hCG) resulted in a similar effect on mammary differentiation as pregnancy. Control virgin mice were treated with hCG 20 U/day for 8 days and then the glands were histologically analyzed. As expected, hCG treatment resulted in a tremendous increase in the formation of alveoli (**E & F**). Although, the magnitude of MRG effect is less than that of hCG on the formation of alveoli, the MRG-induced formation of alveoli is compatible to that of hCG and is significant vs. the control virgin mice.



**Fig. 6.** Histological analysis of alveoli structure. Third pairs of mouse whole thoracic mammary glands were isolated from 7-week old female virgin mice. All the sections were stained with H&E for histological analysis. **A&B**, control mouse mammary gland. **A**, 2x10, an arrow indicates lymph nodes. **B**, 10x10, an arrow indicates ductal structure. **C&D**, MMTV/MRG mouse mammary gland. **C**, 2x10. **D**, 10x10, arrowheads indicate alveolar structure. **E&F**, mammary gland from hCG treated mouse. Six-week old mice were treated with hCG 20 U/day for 8 days and then the glands were isolated for histological analysis. **E**, 2x10. **F**, 10x10, arrowheads indicates alveolar structure.

*C & D. To determine if the mammary tumor incidence and multiplicity are decreased in the MRG transgenic mice (not finished)*

In the early of 2003, the small animal facility in our institution where our MMTV/MRG mice are housed got virus infection. A mandatory non-breeding policy was enforced for the animals including our MMTV/MRG mice in the infected facility. During 6-month non-breeding period, we lost two of our strongest MMTV/MRG strains. Rather than continuing on another MMTV/MRG strain, which has the lowest MRG expression in mammary gland, we decided to start over again by establishing new MMTV/MRG mice. We are currently on the characterization of these new MMTV/MRG mice. The proposed studies in the original Task C & D will be started this early summer after we characterize the phenotype of the new MMTV/MRG mice. This is an unexpected accident and we will do whatever it takes to try to finish the study by early 2005.

### III. KEY RESEARCH ACCOMPLISHMENTS:

1. MRG protein expression was associated with human mammary gland differentiation, with the highest expression observed in the differentiated alveolar mammary epithelial cells from the lactating gland.
2. Transfection of human breast cancer cells with MRG gene resulted in differentiated phenotypes.
3. Treatment of mouse whole mammary gland in organ culture with purified recombinant MRG protein induced gland differentiation with  $\beta$ -casein expression and differentiated morphology.
4. Overexpression of MRG in the mammary gland of transgenic mice resulted in  $\beta$ -casein expression and an increased formation of lobulo-alveoli in the gland.

### IV. REPORTABLE OUTCOMES AND CONCLUSIONS:

There is an increasing public interest in the impact of pregnancy-induced differentiation on breast cancer incidence. As a hormonally related process, the evidence is now convincing, and it is widely accepted that early pregnancy and breastfeeding reduce the risk of breast cancer. Manipulation of pregnancy-like differentiation is a novel and broad approach for breast cancer prevention. Little is known about the regional and developmental expression of locally acting differentiating factors in the mammary epithelium during pregnancy. Within this content, we have previously identified, cloned, and characterized a novel growth inhibitor and a fatty acid binding protein MRG in human mammary gland.

We now report that MRG, which is highly expressed in differentiated lactating human mammary gland, induces the functional differentiation of mammary epithelial cells in cell culture and in mammary gland organ culture. We also investigated the *in vivo* role of MRG in mammary gland development and differentiation in the MMTV/MRG transgenic mice model. We demonstrated that 1) exogenous expression of MRG resulted in differentiated gland morphology with increased formation of lobulo-alveoli-like structure; and 2) consistent with the morphological change, MRG stimulated milk protein  $\beta$ -casein expression in the gland of the transgenic mice. MRG is a candidate mediator of the differentiating effect of pregnancy and lactation on breast epithelial cells and up-regulation of MRG expression in young nulliparous females may mimic pregnancy- and lactation-induced mammary gland differentiation and prevent breast cancer incidence. MRG can also be used as a surrogate endpoint to guide for breast cancer prevention.

### V. References:

1. H. Ji, Y.E. Liu, T. Jia, M. Wang, J. Liu, G. Xiao, B.K. Joseph, C. Rosen and Y.E. Shi. Identification of a breast cancer-specific gene, BCSG1, by direct differential complementary DNA sequencing. **Cancer Res.**, 57: 759-764, 1997.
2. Y. Eric Shi, Jian Ni, Guowei Xiao, Yiliang E. Liu, Alexander Fuchs, Guoliang Yu, Jeffery Su, John M, Cosgrove' Lily Xing, Mei Zhang, Jiyou Li, Bharat B. Aggarwal, Anthony Meager, and Reiner Gentz. Antitumor activity of the novel human breast cancer growth inhibitor MRG. **Cancer Res.**, 57 (15): 3084-3091, 1997.
3. Bielka H, Grosse R, Bohmer F, Junghahn I & Binase B. Inhibition of proliferation of Ehrlich ascites carcinoma cells is functionally correlated with reduced activity of the cytosol to stimulate protein synthesis. **Biomed. Biochim. Acta.**, 45: 441-445, 1986.
4. Böhmer FD, Mieth M, Reichmann G, Taube C, Grosse R & Hollenberg MD. A polypeptide growth inhibitor isolated from lactating bovine mammary gland (MDGI) is a lipid-carrying protein. **J. Cell Biochem.**, 38: 199-204, 1988.

5. Unterberg C, Borchers T, Hojrup P, Roepstorff P, Knudsen J & Spener F. Cardiac fatty acid-binding proteins. Isolation and characterization of the mitochondrial fatty acid-binding protein and its structural relationship with the cytosolic isoforms. *J. Biol. Chem.*, 265: 16255-16261, 1990.
6. Brandt R, Pepperle M, Otto A, Kraft R, Bohmer FD & Grosse R. A 13-KD protein purified from milk fat globule membranes is closely related to a mammary derived growth inhibitor. *Biochemistry*, 27: 1420-1425, 1988.
7. Yang Y, Spitzer E, Kenney N, Zschiesche W, Li M, Kromminga A, Muller T, Spener F, Lezius A & Veerkamp JH. Members of the fatty acid binding protein family are differentiation factors for the mammary gland. *J. Cell. Biology*, 127: 1097-1108, 1994.
8. Kurta A, Vogel, Funa K, Heldin CH & Grosse R.. Developmental regulation of mammary-derived growth inhibitor expression in bovine mammary tissue. *J. Cell Biol.*, 110 (5): 1779-1789, 1990.
9. Binas B, Spitzer E, Zschiesche W, Erdmann B, Kurtz A, Muller T, Niemann C, Blenau W & Grosser R. Hormonal induction of functional differentiation and mammary-derived growth inhibitor expression in cultured mouse mammary gland explants. *In Vitro Cell Dev. Biol.*, 28A: 625-634, 1992.
10. Li M, Spitzer E, Zschiesche W, Binas B, Parczyk K & Grosse R. Antiprogesterins inhibit growth and stimulate differentiation in the normal mammary gland. *J. Cell Physiol.*, 164: 1-8, 1994.
11. Yang Y, Spitzer E, Kenney N, Zschiesche W, Li M, Kromminga A, Muller T, Spener F, Lezius A & Veerkamp JH. Members of the fatty acid binding protein family are differentiation factors for the mammary gland. *J. Cell. Biology*, 127: 1097-1108, 1994.
12. Y. Eric Shi. Correspondence re: Y.E. Shi et al., Antitumor activity of the novel human breast cancer growth inhibitor, mammary-derived growth inhibitor-related gene, MRG. **Cancer Res.**, 58: 4015-4017, 1998.
13. Böhmer FD, Mieth M, Reichmann G, Taube C, Grosse R & Hollenberg MD. A polypeptide growth inhibitor isolated from lactating bovine mammary gland (MDGI) is a lipid-carrying protein. *J. Cell Biochem.*, 38: 199-204, 1988.
14. Specht B, Bartetzko N, Hohoff C, Kuhl H, Franke R, Borchers T & Spener F. Mammary derived growth inhibitor is not a distinct protein but a mix of heart-type and adipocyte-type fatty acid-binding protein. *J. Biol. Chem.*, 271: 19943-19949, 1996.
15. Treuner M, Kozak CA, Gallahan D, Grosse R & Muller T. Cloning and characterization of the mouse gene encoding mammary-derived growth inhibitor/heart-fatty acid-binding protein. *Gene*, 147(2): 237-242, 1994.
16. Kurtz A, Spitzer E, Zschiesche W, Wellstein A, and Grosse R. Local control of mammary gland differentiation: mammary derived growth inhibitor and pleiotrophin. *Biochem. Soc. Symp.* 63: 51-69, 1998.
17. Borchers T, Hohoff C, Buhlmann C, and Spener F. Heart-type fatty acid binding protein-involvement in growth inhibition and differentiation. *Prostaglandins Leukot. Essent Fatty Acids*, 57: 77-84, 1997.
18. Bass NM and Manning JA. Tissue expression of three structurally different fatty acid binding proteins from rat heart muscle, liver and intestine. *Biochem. Biophys. Res. Commun.* 137: 929-935, 1986.
19. Billich S, Wissel T, Kratzin H, Hahn U, Hagenhoff B, Lezius AG and Spener F. Cloning of a full-length complementary DNA for fatty-acid-binding from bovine heart. *Eur. J. Biochem.* 175: 549-556, 1988.
20. Zschiesche W, Kleine AH, Spitzer E, Veerkamp JH and Glatz JF. Histochemical localization of heart-type fatty-acid binding protein in human and murine tissues. *Histochem. Cell. Biol.*, 103: 147-156, 1995.

21. Mingsheng Wang, Yiliang E. Liu, Jian Ni, Banu Aygun, Itzhak D. Goldberg, Y. Eric Shi. Induction of mammary differentiation by MRG that interacts with  $\omega$ -3 fatty acid on growth inhibition of breast cancer cells. *Cancer Res.* 60: 6482-6487, 2000.
22. Feng L, Hatten ME and Heintz N. Brain lipid-binding protein (BLBP): a novel signaling system in the developing mammalian CNS. *Neuron* 12 (4): 895-908, 1994.
23. Anton ES, Marchionni MA, Lee KF and Rakic P. Role of GGF/neuregulin signaling in interactions between migrating neurons and radial glia in the developing cerebral cortex. *Development* 124 (18): 3501-10, 1997.
24. Huynh H, Alpert L and Pollak M. Silence of the mammary-derived growth inhibitor (MDGI) gene in breast neoplasms is associated with epigenetic changes. *Cancer Res.*, 56: 4865-4870, 1996.
25. Kurta A, Vogel F, Funa K, Heldin CH & Grosse R. Developmental regulation of mammary-derived growth inhibitor expression in bovine mammary tissue. *J. Cell Biol.*, 110 (5): 1779-1789, 1990.
26. Huynh H, Larsson C, Narod S and Pollak M. Tumor suppressor activity of the gene encoding mammary-derived growth inhibitor. *Cancer Res.*, 55: 2225-2231, 1995.
27. Theile M, Seitz S, Arnold W, Jandrig B, Frege R, Schlag PM, Hansch W, Guski H, Winzer KJ, Barrett JC and Scherneck S. A defined chromosome 6q fragment (at D6S310) harbors a putative tumor suppressor gene for breast cancer. *ncogene*, 13: 677-685, 1996.
28. Bieche I, Champeme MH & Lidereau R. A tumor suppressor gene on chromosome 1p32-pter controls the amplification of MYC family genes in breast cancer. *Cancer Res.*, 54: 4274-4276, 1994.
29. Xu LZ, Sanchez R, Sali A and Heintz N. Ligand specificity of brain lipid-binding protein. *J. Biol. Chem.* 271 (40): 24711-24719, 1996.
30. Kaizer F, Boyd NF, Kriukov V, Trichler D: Fish consumption and breast cancer risk: an ecological study. *Nutr Cancer* 12: 61-68, 1989.